Standard Operating Procedure Fish Necropsy and Tissue Histologic Preparation Dr. Jan Spitsbergen, DVM, Ph.D., Diplomate American College of Veterinary Pathologists, September 10, 2008

Necropsy and Histopathology Procedure for Small Fish (6 cm Total Length or Less)

Euthanasia and Tissue Preparation for Histopathologic Study. Fish will be euthanized by immersion in a lethal concentration of tricaine methane-sulfonate (MS222) in well water. Length, weight and any gross lesions will be recorded. Gross lesions will be photographed if unique. The caudal peduncle will be cut off, the abdomen opened ventrally and gas bladder punctured prior to placing the carcass into buffered zinc formalin. Following 24 hr fixation, fish will be decalcified in Cal X II (formic acid-formalin solution) for 24-48 hours if needed. Samples will be dehydrated in graded ethanols, embedded in paraffin, and saggital 4 micrometer step sections will be cut from the left side of the fish. 9 step sections from each fish will be mounted on 3 glass slides, with sections mid-eye level on the left to mid-eye level on the right (Fournie et al, 1996). Grossly visible lesions may be embedded in orientations best suited to study the particular lesions. Sections will be stained routinely with hematoxylin and eosin (H&E). If preparation of plastic-embedded tissue is warranted based on lesions seen in paraffin-embedded tissue or in other assays, tissue fixed in zinc formalin will be embedded in Historesin and 1 micron saggital histologic sections will be prepared from the midline region of fish.

Alternative Histologic Sectioning Method (more efficient and leaves tissue in the block for recuts for special stains or immunohistochemistry). Prior to embedding of fish in paraffin, the fish will be cut in half saggitally just to the left of midline using a razor blade. Both halves of the fish will be placed cut side down in the histology cassette (or cassettes for larger fish). Then histologic sections will be cut and saved for staining after facing the block (midline), at a level just medial to the eyes, and at a level through the middle of each eye. This method generates more histologic sections on fewer microscope slides and leaves more tissue in the paraffin block for recuts.

Fournie, J. W., W. E. Hawkins, R. M. Krol, and M. J. Wolfe. 1996. Preparation of whole small fish for histological evaluation. Pages 577-588 in G. M. Ostrander, ed. *Techniques in Aquatic Toxicology*. Lewis Publishers, Boca Raton, FL.

http://zebrafish.org/zirc/health/diseaseManual.php

(histo methods in appendix of Disease Manual on ZFIN)

Necropsy Procedure for Larger Fish (Over 6 cm Total Length). The fish will be euthanized by immersion in a lethal concentration of tricaine methane-sulfonate (MS222) in well water. Length, weight and any gross lesions will be recorded. Gross lesions will be photographed if unique. Scales or otoliths will be preserved for aging if desired. A midline incision will be made in the abdomen from heart to anus and all major organs will be examined grossly. Representative pieces, less than 1 cm thick, of all major organs will be fixed in buffered zinc formalin, with tissues placed in at least 10X their volume of fixative for 24 hours. Any gross lesions will be preserved for histology. Tissue samples will be dehydrated in graded ethanols, embedded in paraffin, and sectioned at 4

micrometers thickness. Sections will be stained routinely with hematoxylin and eosin (H&E). Any gross lesions will be correlated with associated histologic lesions.

Davidson's Solution

- 95% ethanol 300 ml
- formalin 200 ml
- glacial acetic acid 100 ml
- distilled water 300 ml

Dietrich's Fixative

- 95% ethanol30 mL
- formalin10 mL
- glacial acetic acid 2 mL
- distilled water58 mL
- 100 mL total

Millonig's Buffer for Electron Microscopy

Solution A 2.62 % NaH_2PO_4

Solution B 2.52 % NaOH

Working solution:

Mix 83 ml Solution A with 17 ml Solution B.

Adjust pH to 7.4 with 10 N NaOH or concentrated HCl.

For transmission electron microscopy of fish tissues, we generally prepare 4% glutaraldehyde in Millonig's buffer. Fix small pieces of tissue at room temperature for 2 h, then hold overnight at 4 ^{0}C. Transfer to Millonig's buffer without glutaraldehyde.

Cytophaga Medium (for Gliding Bacteria)

- tryptone 0.5 g
- yeast extract 0.5 g
- sodium acetate 0.2 g
- beef extract 0.2 g
- agar 11.0 g

add above to:

- distilled water 1 liter
- autoclave and pour into sterile petri dishes

Leishman's Giemsa Stain

This stain is useful for examining protozoa, bacteria and cell morphology in tissue imprints and blood smears. Pre-made commercial products, such as Diff-Quik (Dade Behring AG, Newark, DE) http://www.dadebehring.com/ are nearly equivalent.

Leishman's
Add 1 g Leishman's stain to 500 ml absolute methanol and filter.

Giemsa Stock Solution
Add 1 g powder to 66 ml to glycerol and heat at 60 ^{0}C for one hour. Then add 66 ml of absolute methanol and filter. Store at 40C.

Phosphate Buffer Solutions

- Solution A - 31.20 g NaH_2PO_4 , 2 H_20 in 1 L distilled water
- Solution B - 53.65 g Na_2HPO_4 , 7 H_20 in 1 L distilled water

Giemsa Working Solution
To prepare phosphate buffer, mix 73.5 ml solution A with 26.5 ml solution B. Then add 100 ml distilled water. Add 3.5 ml stock solution to 50 ml phosphate buffer (pH 6.4). Make fresh for each use.

Staining Procedure:

1. Prepare smear or imprint and allow to dry for about 1/2 hour.
2. Fix in methanol for 2-5 min.
3. Stain in Leishman's for 2-3 min.
4. Stain in Giemsa for 10-12 min.
5. Rinse in distilled water for about 1 min.
6. Air dry.

Adult Zebrafish Histology

Fixation for histology

- Fish should still be alive just prior to fixation.
- Ice the fish to kill them. Cut off the tail behind the anus. Slit open the body cavity along the belly.
- Use 15 mL fixative per 1-2 fish in a small vial.
- To ensure uniform and complete fixation, fix for three days on rotor, or other agitating device.
- Store fish in Dietrich's fixative at room temperature

Processing for Histology

Adult zebrafish are often difficult to section without first softening their skin and scales. The day before processing, transfer the fixed fish to 5% trichloroacetic acid (TCA) in Dietrich's fixative, and place on rotor overnight. TCA will soften the scales and skin, presumably by cleaving the keratin proteins. In addition, TCA will decalcify, softening the bones. Unfortunately, TCA is a fairly strong acid and will corrode the tissue processor. The fish must be thoroughly rinsed to remove any TCA before the fish can be processed.

PROTOCOL:
Transfer fish to 5% TCA in Dietrich's fixative. Place on rotor overnight. The next day, replace TCA-containing Dietrich's with 70% ethanol and place on rotor for 10 minutes. Repeat 2xs for a total rinse time of 30 minutes. Replace last rinse with fresh 70% ethanol. The fish are then bisected along their length before they

are processed. Using a fresh razor blade, make the cut parallel to and on the left side of the spinal cord. The bisected fish are now ready to load into cassettes for processing.

Processing Schedule for Large Adults (4-5 months or older):

- 70% ethanol 10 minutes
- 70% ethanol 40 minutes
- 80% ethanol 1 hour, 10 minutes
- 95% ethanol 2 X 1 hour, 10 minutes
- 100% ethanol 2 X 1 hour, 10 minutes
- xylene 1 hour, 30 minutes
- Clear-Rite 3 (from Richard-Allen Scientific) 1 hour, 30 minutes
- Paraplast/Paraplast Plus* (50:50) 2 X 1 hour
- Paraplast 1 hour
- Paraplast 45 minutes
- Embed in TissuePrep-2 Embedding Media (Fisher Scientific), placing cut sides of both halves of the fish down.
- *Paraplast Plus contains DMSO. Care should be exercised when handling used Paraplast Plus as the DMSO can carry residual xylene across the skin.

Processing Schedule for Small Adults (1-3 months):

- 70% ethanol 10 minutes
- 70% ethanol 50 minutes
- 80% ethanol 50 minutes
- 95% ethanol 2 X 50 minutes
- 100% ethanol 2 X 50 minutes
- xylene 1 hour, 10 minutes
- Clear-Rite 3 (from Richard-Allen Scientific) 1 hour, 10 minutes
- Paraplast/Paraplast Plus* (50:50) 2 X 1 hour
- Paraplast 1 hour
- Paraplast 45 minutes
- Embed in TissuePrep-2 Embedding Media (Fisher Scientific), placing cut sides of both halves of the fish down.

- *Paraplast Plus contains DMSO. Care should be exercised when handling used Paraplast Plus as the DMSO can carry residual xylene across the skin.

Processing Schedule for Young Fish (1 week-1 month)

- Fish younger than 3 weeks can be pre-embedded in Histogel, available from Fisher Scientific, Richard Allan Scientific or Lab Storage Systems.
- 70% ethanol 2 X 20 minutes
- 80% ethanol 30 minutes
- 95% ethanol 2 X 30 minutes
- 100% ethanol 2 X 30 minutes
- xylene 40 minutes
- Clear-Rite 3 (from Richard-Allen Scientific) 40 minutes
- Paraplast/Paraplast Plus* (50:50) 2 X 40 minutes
- Paraplast 40 minutes
- Paraplast 15 minutes

If you want serial sections, embed in Paraplast as this paraffin provides for better ribboning.

Sectioning Fish

Cut 7 µm sections with a high-profile disposable blade (AccuEdge blades -- made by Sakura-Finetek, sold through VWR -- are reportedly the best) . If the large fish are difficult to cut, soak the block face in ice-cold Mollifex. Usually one to two hours is sufficient but an overnight soak is fine for stubborn samples.

Sections of gills, spinal cord and internal organs will be needed for proper pathological evaluation. Because the fish has been cut to one side of the spinal cord, you will be cutting toward the midline on one half of the fish, and away from the midline on the other. With any luck, you will need to collect only one ribbon containing gills (one bisected half) and spinal cord (the other bisected half), along with a nice sampling of internal organs. Samples of the skin or fins may also be requested, depending on the reported symptoms.

Mollifex: 54 mL 95% ethanol, 10 mL glycerol, 36 mL water

Store fixative at room temperature.

H&E Protocol for Adult Zebrafish

The following is a staining protocol perfected by Karen Larison at ZIRC that we routinely use for zebrafish histopathology. Any water stage is a good stopping point if you had to do something part way through the procedure. Keep the slides wet throughout the procedure.

- Clear-Rite 3 3X 3min
- 100% ethanol (absolute) 3X 1 min
- 80 % ethanol 30 sec
- 50 % ethanol 30 sec
- 30 % ethanol 30 sec
- Rinse in slow running water 30 sec
- Harris' Hematoxylin * 4.5 min
- Rinse in slow running distilled water till stain rinsed out ~4 min
- Decolorize in 0.1% Acid alcohol** 45 sec
- Rinse in distilled water 15 sec
- Lithium carbonate neutralizing station** * 8 dips

At this point the sections will look blue.

- Rinse in slow running water minimum of 5 min
- 95 % ethanol 30 sec
- 95% ethanol 30 sec
- eosin Y - phloxine B solution**** 30 sec
- 100 % isopropanol***** 6X 15 sec
- xylene 3X 15 sec
- Coverslip with permount.

* Filter Hematoxylin before each use. Make fresh batch every 2-3 months.
** 200 ul concentrated HCl in 200 mL 70% ethanol
*** Water and lithium carbonate (approx 2 tablespoons, supersaturated)
**** Make fresh eosin Y- phloxine B solutions from stock every 1-2 weeks.
***** Use fresh isopropanol for the 6th wash. Discard the 1st (and pinkest) isopropyl. Rinse in isopropyl waste container after each staining session. Store other used rinses in bottles 1-5 (pinkest to clearest) for future use.

Harris' Hematoxylin (Theory & Practice of Histological Techniques, Bancroft & Stevens, 4th edition, 1996, pp 101-102)

- Hematoxylin 2.5 g
- Absolute alcohol 25 ml
- Potassium alum 50 g
- Distilled Water 500 mL
- Sodium iodate 0.5 g
- glacial acetic acid 20.0 mL

The hematoxylin is dissolved in the absolute alcohol, and then added to the alum, which has previously been dissolved in the warm distilled water in a 2-liter flask. The mixture is rapidly brought to a boil and sodium iodate is then slowly and carefully added. The stain is rapidly cooled by plunging the flask into cold water or into a sink containing chipped ice. When the solution is cold, the acetic acid is added, and the stain is ready for immediate use.

Eosin Y - Phloxine B solution (from Gayle Callis)

- 1% eosin Y (aq) 25.0 mL
- 1% phloxine B (aq) 2.5 mL
- 95% ethanol 195.0 mL
- glacial acetic acid 1.0 mL

Acid Fast Stain for Mycobacteria

1. Deparaffinize and rehydrate to water	
2. Clear-Rite 3	3 times for 3 min each
3. 100% ethanol	3 times for 1 min each
4. 80% ethanol	30 sec
5. 50% ethanol	30 sec
6. 30% ethanol	30 sec
7. Rinse is slow running water	30 sec
8. Carbol-fuschin	30 min
9. Rinse in distilled water	
10. 1% glacial acetic acid in 70% ethanol	about 1.5 min, depending on tissue*
11. Rinse in running tap water	10 min
12. Methylene blue	short dip

13. Rinse in running tap water	5 min
14. 95% ethanol	6 dips
15. 100% ethanol	2 min
16. 100% ethanol	5 min
17. xylene	3 min (repeat 3 times)

Stock Solutions

Carbol-fuschin

Basic fuschin	1 g
Melted phenol	5 g
100% ethanol	10 ml
Distilled water	100 ml

Filter before use, can be re-used

Methylene Blue

Methylene blue	0.14 g
95% ethanol	10.0 ml
Tap water	90 ml

Dissolve stain in ethanol, then add water. Discard after use.

* Step 10. This step is subjective. The goal is to decolorize tissues while bacteria retain the stain.